Antibody Incubation for Microwestern Array

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Validated by / Date:

NOTE: This protocol was inspired by that written by Mark Ciaccio (Ciaccio MF, Wagner JP, Chuu CP, Lauffenburger DA, Jones RB (2010) Systems analysis of EGF receptor signaling dynamics with microwestern arrays. Nat Methods 7:148–155; Youtube videos on MWA, www.youtube.com/watch?v=0iUhoWL1lC0).

1) Prepare 1 L of 10X Wash Buffer
    a. Weigh 24.2 g Tris base (Sigma, #93350) and 80 g NaCl (Fluka, #71383); dissolve in 900 mL of milliQ water.
    b. Adjust pH to 7.6 with 12M HCl (Ricca, #7274-16) and bring final volume to 1 L. Store at 4^{o}C, good for 1 year.
2) Prepare 50 mL 1X wash buffer just prior to use by adding 5 mL 10X wash buffer to 45 mL milliQ water.
3) Prepare Nitrocellulose (NC) that contains the samples for primary antibody incubation.
    a. Wearing gloves, remove NC from the blotter pad/gel/NC/blotter pad sandwich in the Criterion Blotter gel holder (see SOP A-14.0, “Wet Transfer to Nitrocellulose for Microwestern Array.” Steps 7-9)
        i. Place blotter gel holder cassette red side down, remove pad and gel exposing NC with samples facing up. Mark A1 corner with notch (see Figure 1). A1 corner is defined by the specific workplate used to print samples. (See Step 7, Fig.1 in SOP A-11.0, “Printing of Samples for Microwestern Array).
        ii. Lift NC off underlying pad and place in a container (such as the plastic lid from a pipette tip box) sample side up. Add 50 mL 1X Wash buffer, enough to cover NC, wash with gentle rocking for 5 minutes at room temperature. Place aluminum foil over container to block ambient light.
        iii. Remove wash buffer and add 50 mL, enough to cover NC, of Odyssey Blocking Buffer (Licor, #927-50000) for 30 minutes to 1 hour with gentle rocking at room temperature. Block ambient light with foil.

4) Prepare primary antibodies. Preparation will depend on the type of hybridization plate used and the concentration of the antibodies. A 24 well plate uses 0.5 mL per well; a 96 well plate uses 0.10 mL per well. Dilute in Odyssey Blocking Buffer.
    a. Use an antibody concentration that has been validated for a specific antibody. Antibodies are validated for a specific cell type under certain conditions with a minimum of 4 points out of 6 producing a discernible signal covering a log scale and with a linear R^2 value of at least 0.95.
5) After removal from blocking buffer, trim NC to allow for proper alignment within the hybridization plate. (Example below is based on a 24 well plate setup.) The following figures show how the NC is trimmed along the x axis using the "cut lines" (leaving a length of 118 mm, Fig. 1a.) and along the y axis to a point 77 mm from the bottom of the NC (Fig. 1.b.) to fit into the Hybridization Plate (Fig. 2.a.) (The Gel Company, #MWA100-24-1) so that the "alignment line," Fig. 1a, lies on top of the A6-D6 gasket line (Fig. 2.b.).
    a. Figure 1a Alignment and cut line markers along columns 1 – 6 axis (M.W. markers from LiCor #928-40000, printed on the gel at the same time as samples)

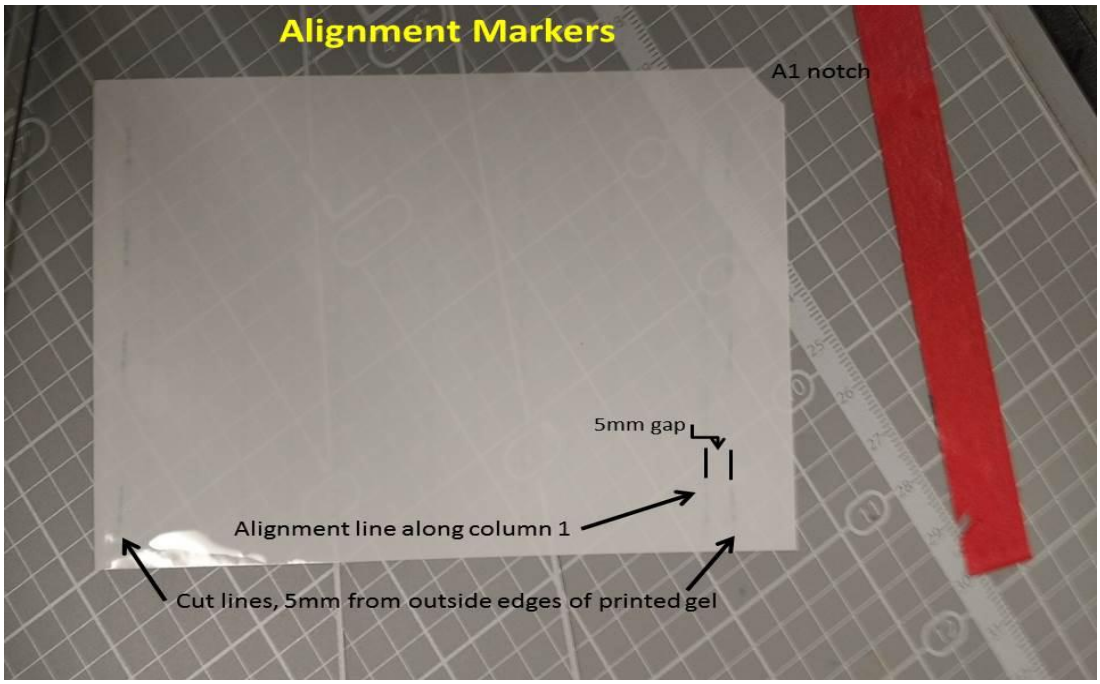
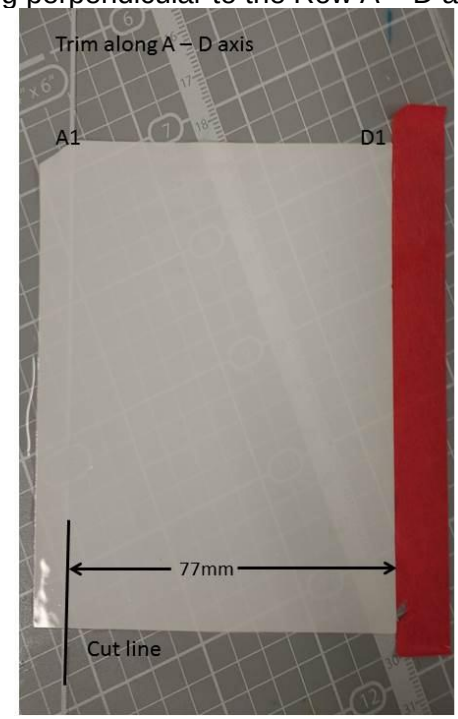


Figure 1.b. Trimming perpendicular to the Row A – D axis



6) Place NC sample side up in plate, carefully aligning the printed areas with the locations of the hybridization wells. Alignment line (see Fig. 1.a. above) should be positioned along gasket line of the hybridization plate, 5mm from plate edge.

Figure 2.a. Hybridization Plate measurements and position markers

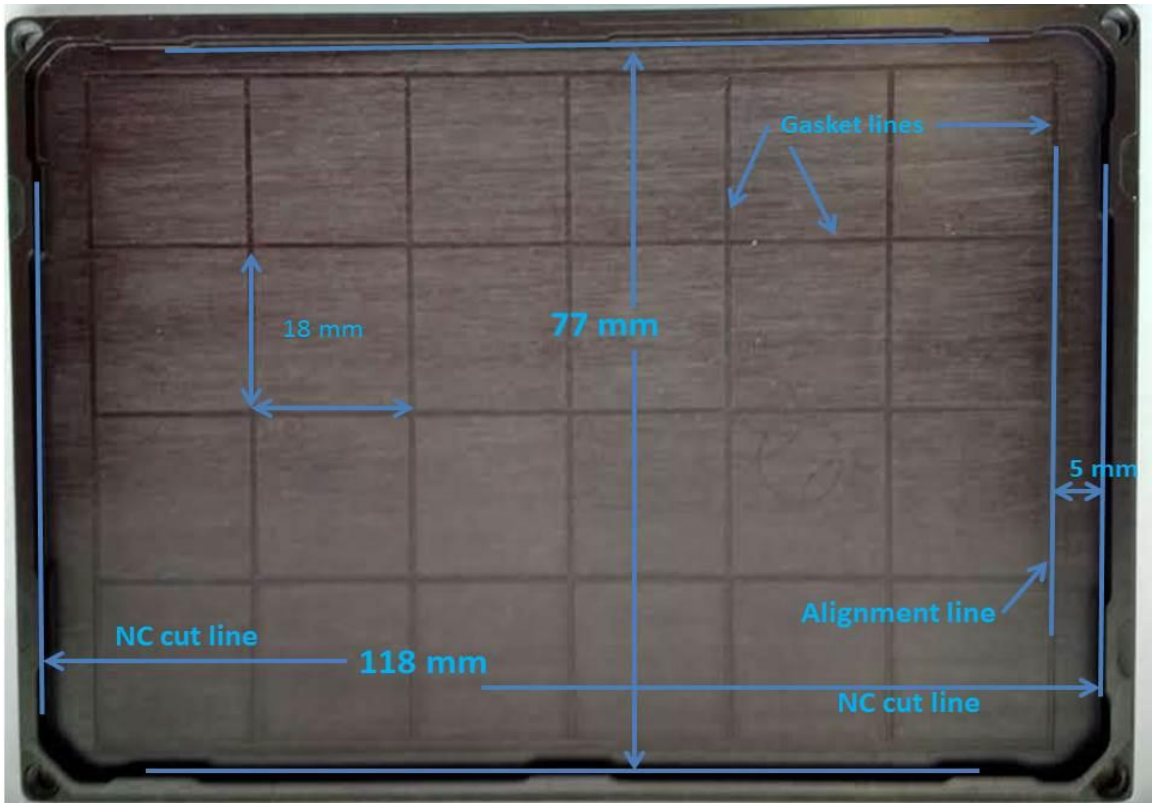
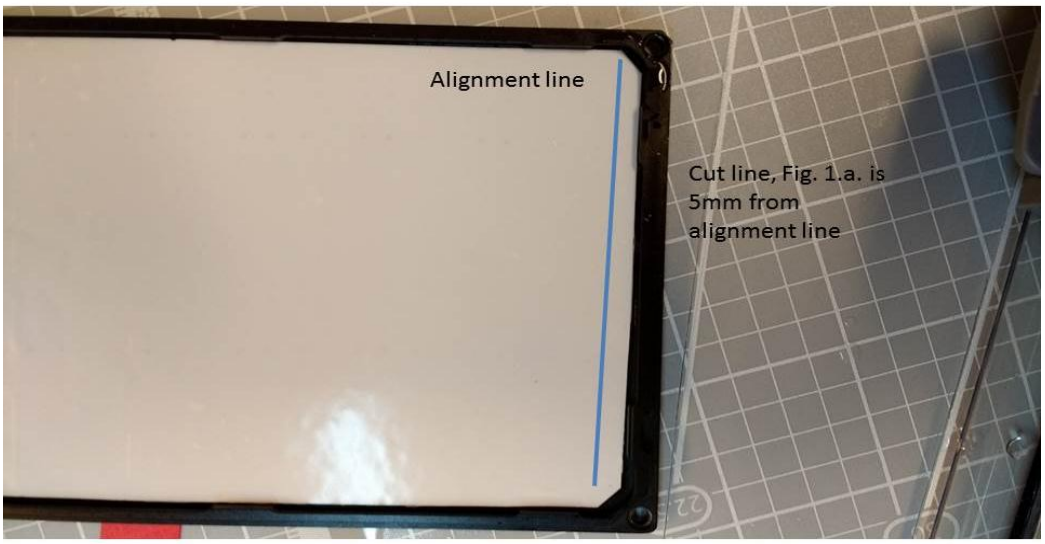


Figure 2.b. NC Alignment Line along Gasket line in Hybridization Plate



7) Place the top of Hybridization plate on the wet NC firmly tightening screws by hand to form the hybridization wells.
    a. Ensure NC membrane does not dry out during this process. Drying out of NC is indicated by a loss of translucency, becoming opaque.
8) Pipette antibodies appropriately into wells carefully, using a micropipette, as dictated by the plate map for antibody-well mapping. Antibodies are diluted in Odyssey Blocking Buffer. to a concentration that has been validated for a specific antibody. See table in Metadata.
    A 24 well plate uses 0.5 mL per well; a 96 well plate uses 0.10 mL per well.
9) Cover plate with one sheet of MicroAmp Optical adhesive film (Applied Biosystems, #4313663) to avoid evaporation.
10) Incubate overnight with gentle rocking at 4^{o}C, blocking ambient light with aluminum foil
11) Aspirate antibody solution from each well.
12) Wash NC five times while protecting from ambient light.
    a. Mix 1x Wash buffer with Tween 20 (Sigma, #P1379) to make a 0.1% solution (100µL of Tween 20 + 100 mL of 1X Wash buffer)
    b. Add 0.5 mL of the 1X wash buffer + Tween to each well with a micropipette
    c. Incubate at room temperature with gentle rocking for five minutes.
    d. Aspirate wash from each well.
13) Prepare Li-Cor near infrared secondary antibodies.
    a. Prepare secondary antibody buffer fresh prior to use: Li-Cor Blocking buffer 20%; 10X Wash buffer 10% and 70% milliQ water.
    b. Use an appropriate combination of 680/700 IR-conjugated and 800 IR-conjugated secondary antibodies depending on the species of primary antibody used. Dilute antibodies from 1/5000 (1 µl stock in 5 mL of buffer) to 1/20,000 (1 µl stock in 20 mL of buffer).
    c. We use goat anti-mouse (Licor IRDye 680RD #926-68070) for the “700,” “red” signal and goat anti-rabbit (Licor IRDye 800CW #926-32211) for the 800 “green” signal. For a 24 well hybridization plate 500 µL is added to each well; for a 96 well hybridization plate add 100 µL.

14) Incubate for 1 hour with gentle rocking at room temperature and protect from light. Protect from light in steps 15 – 18.
15) While still in the hybridization plate, wash membrane three times with 0.5 mL per well for a 24 well hybridization plate and 0.1 mL per well for a 96 well hybridization plate with 1x Wash buffer with 0.1% Tween 20 (see step 12),
16) Remove NC from plate and wash once in 1x Wash buffer with 0.1% Tween (see step 11) in a container such as a polypropylene pipet tip box top for 5 to 10 minutes. Use enough volume to cover the NC; usually 50 mL is sufficient.
17) Wash twice in 1x Wash buffer without Tween, 50 mL per wash for 5 to 10 minutes.
18) Place NC membrane on clean paper towel and allow to dry completely at room temperature
19) Place membrane sample side down on the scanning surface of the Li-Cor Odyssey scanner. Place a clean piece of glass over the NC to keep it flat.
20) Scan using a resolution of 42 microns/pixel and high quality if using the LiCor Odyssey scanner. If using the Li-Cor Odyssey CLx scanner set at “Auto.” See SOP A-16.0 for details of analysis.